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# ANTIFUNGAL ACTIVITY OF HYDROGEN PEROXIDE BASED DISINFECTANT BIOXIL

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**ABSTRACT**

Antifungal activity of the Bioxil containing hydrogen peroxide as an active agent has been studied. The mentioned species act as indicators of microbiological quality of fruit juices and hygienic condition of juice processing industry. Inhibition activity of the mentioned disinfection agent has been studied *in vitro* against different yeast strains: *Rhodotorula rubra* J-120 and *Saccharomyces cerevisiae* J-200 and their mix culture. For the evaluation of antifungal efficacy of the Bioxil suspension and surface tests were used. Relation between antifungal activity of Bioxil, its concentrations, yeast species and contact times was established. Bioxil containing 1% and 3% of $H_2O_2$ has shown unequal inhibition activity against different yeast strains. Influence of washing and disinfection steps on the level of contamination of conveyor and working surfaces by yeasts and has been investigated. In the processing conditions, after treatment of conveyors and working surfaces, without preliminary washing, lower inhibition activity of the Bioxil has been occurred.

**Keywords:** Yeast, hydrogen peroxide, antifungal activity, Bioxil

## INTRODUCTION

Hydrogen peroxide and its compounds are the well known biocides, which include huge amount of peroxides' derivatives and salts. Hydrogen peroxide ($H_2O_2$) is a chemical compound mostly used in sterilization. Amongst other benefits, it is not toxic at residual concentrations and allows for a quick recovery treatment **(Von Bockelmann, 1972).** Hydrogen peroxide has been shown to possess a wide spectrum of antimicrobial activity, in that it is active against bacteria, yeasts, fungi, viruses and spores. Normally, these protocols make use of a 0.25-3% $H_2O_2$ concentration range **(Tschernjawskaja; Belowa, 1990)**. The efficacy of hydrogen peroxide depends on many factors, for example: concentration, pH, temperature, reaction time, use in combination with physical agents. Moreover, it depends on bacterial/viral concentration, the microbial species under consideration and their biological phase (e.g., spore or vegetative *status*), the presence of organic substances, the nature of the surface to be treated (presence of pores, micro-cracks) and bacterial genetic proprieties **(Feuerstein *et al*., 2006).** Yeasts are the most significant group of microorganisms associated with spoilage of soft drinks and fruit juices **(Hocking, 2001; Kurtzman, 2006).** Fruits juices are commonly contaminated by spoilage yeasts resulting in the loss of quality (**Stratford M. , 2007**).

Spoilage yeasts, such as *Saccharomyces cerevisiae*, *Candida lipolytica*, *Zygosaccharomyces bailii* and *Rhodotorula rubra* are widely distributed in juice processing plants and possess high resistance to chemical preservatives (**Valsaraj et all., 2012**). In most cases hygienic conditions of processing are determined by the presence of following yeast species: *Aerobasidium pullulans*, *Candida solani*, *Rhodotorula glutinanas*, *R. rubra* (**Tudor, 1993**). Their progression is governed by the ability to grow at low temperatures, low water activity, high salt concentrations, fermentation/assimilation of lactose, production of lipolytic and proteolytic enzymes and utilization of lactic and citric acids (**Baird-Parker, *et al*., 1998;**). *Saccharomyces* species are widely distributed ecologically, and are widely used industrially; it is inevitable that they will contaminate many foods and beverages. *S. cerevisiae* is used commonly as the test organism in efficacy studies of disinfectants **(Collinson, *et al*., 1992)**. There is some evidence that ascospores of *S. cerevisiae* are more resistant than vegetative cells to hydrogen peroxide and quaternary ammonium compounds, but not to peracetic acid **(Romano, 1985).**

Poor cleaning and sanitation of processing equipment provide another risk factor that contributes significantly to outbreaks of food and beverage spoilage by yeasts, and good effective practices are essential to prevent or minimize such occurrences. There are few published studies concerning efficiency of disinfecting agents against spoilage yeasts. Nowadays, it is priority for food-processing industries to apply well cleaning and sanitation programs to minimize the risk of biofilm contamination (**Gibson et al. 1999**). Yeasts in biofilms are more resistant to biocidal agents (**Campana, 2002**). Some yeast species for example form genus *Rhodotorula*, *Candida*, *Geotrichum* are able to produce exopolymers that additionally strengthen the community of microorganisms (**Simo`es et al., 2010**).

The maintenance of high hygienic standards during the various steps of juice production is essential to prevent the contamination of fruit juices by spoilage yeasts. However, this is not always easy to accomplish because used disinfectants, in order to be effective, should be able to penetrate the extracellular polymeric substances matrix surrounding the biofilms and kill the cells (Sutherland 2001).

The aim of this study was to determine the efficacy of hydrogen peroxide-based disinfectant (Bioxil) against food spoilage yeasts in laboratory and processing conditions.

## MATERIAL AND METHODS

### Yeasts culture

Strains of *Rhodotorula rubra* J-120, *Saccharomyces cerevisiae* J-200, *Kloeckera apiculata* isolated from fruit juice processing were used in this study.

### Preparation of yeasts cell suspension

The yeast strains were grown on glucose yeasts agar (M963, Himedia) plates at 30°C for 2 to 4 days, after which yeast cells were harvested from colonies and suspended in sterile distilled water. The concentrations of yeasts cells in suspension were adjusted to $10^6$ cfu/ml. Yeasts cell concentration in the suspension was measured with spectrophotometer at 610 nm.

### Hydrogen peroxide solutions

To prepare 1% solution of hydrogen peroxide 7.5 ml 35% solution of hydrogen peroxide is placed into a graduated flask with the volume of 250 ml and then the volume of solution is brought to the niche. To prepare 3% solution of hydrogen peroxide the same procedure is performed with 22.5 ml 30% solution of

hydrogen peroxide. To prepare disinfectant (Bioxil) activators are added to the solution in amounts of 1% relative to hydrogen peroxide content in solution.

**Quantitative suspension method**

The antifungal activity of Bioxil against *R. rubra, S. cerevisiae* and mix culture of yeasts /*R. rubra, S. cerevisiae* and *K. apiculata*/ was studied by quantitative suspension tests in accordance with Directive EN 1650:2008 and EU standard EN 1275:2006. After 5, 15, 30 and 60 min of incubation samples were taken and transferred to tubes containing neutralizer solution (1% of sodium thiosulphate $Na_2S_2O_3$). After neutralization the suspension was transferred on glucose yeast agar plates. The effectiveness of the disinfectant was calculated according to the following equation: $R= N_a \times 10^{-1}/N$, where R is the reduction in viability of cells; $N_a$ – initial quantity of cells in suspension; N-is quantity of cells after testing.

**Surface test**

For the testing the effeciecy of the Bioxil against mix culture of S. *cerevisiae* and *K. apiculata*. by surface test stainless steel coupons (5 cm x 5 cm) were used. 200 µl of sterile strawberry juice was used as soiling agent. 200 µl of mix culture suspension was inoculated onto preliminary contaminated coupons. 5% solution of caustic soda was used for preliminary washing of coupons before treatment my Bioxil. After drying the coupons were treated with disinfectant solution. After 5 and 30 min. of contact time coupons were swabbed with cotton swabs (previously immersed into neutralizer solution/ 1% $Na_2SO_3$). Then serial dilution was performed and diluted samples were plated onto Glucose Yeast Agar and incubated at 30°C for 48-72 hours.

**Testing in processing conditions**

**S**wabbing of juice processing equipment surfaces was carried out in accordance with ISO 18593 standard method. Evaluation of the efficiency of the Bioxil in processing conditions has been done in juice processing factory. Surfaces of conveyor belts have been treated with the mentioned biocide preceded with and without washing by caustic soda. After 15 and 30 minutes of contact time the swabs have been taken / HiCultureTM Transport Swabs, Hi Media/ and transferred to laboratory . Thereafter , swabs have been cultured on Glucose Yeast Agar and incubated at 30°C for 48-72 hours.

**RESULTS AND DISCUSSION**

Antifungal activity of the Bioxil (solutions containing 1% and 3% of hydrogen peroxide) against *S. cerevisiae* and *R. rubra* was studied *in vitro.* Six log reduction of yeast cells *R. rubra* occurred at the end of 5 and 15 min of contact time, (Tab 1). Bioxil has shown high, but not stable inhibition activity against *R. rubra*.

The quantity of yeast cells has increased after 15 min of contact time with Bioxil (1 and 3% $H_2O_2$) and in the end of 60 min of incubation quantity of yeast cells was $7x10^2$ $3x10^2$ cfu/ml respectively. Thus, at the end of 60 minutes of contact time less than 4 log reduction of yeast cells has been occurred.

**Table 1** Determination of inhibitory activity of 1% and 3% aqueous solution of $H_2O_2$ in Bioxil against *R. rubra* J-120 by quantitative suspension test

| Bioxil | Initial quantity of cells in suspension, cfu/ml (N) | Incubation time, min | Quantity of cells after testing ($N_a$) | Reduction in viability, cfu/ml (R) |
|---|---|---|---|---|
| 1% $H_2O_2$ | 2 x $10^6$ | 5 | $5x10^2$ | < $10^4$ |
| | | 15 | 40 | > $10^6$ |
| | | 30 | $2x10^2$ | $10^4$ |
| | | 60 | $7x10^2$ | <$10^4$ |
| 3% $H_2O_2$ | 1.7 x $10^6$ | 5 | 20 | > $10^6$ |
| | | 15 | 25 | > $10^6$ |
| | | 30 | $10^2$ | > $10^4$ |
| | | 60 | $3x10^2$ | < $10^4$ |

The European standard UNE-EN-1650, proposes that in suspension tests a DR of 4.0 log10 (CFU/ml) of the initial yeasts load is good enough to consider that a certain disinfectant has a good fungicidal activity (**Anonymous, 1998**).

Laubsher (**Laubsher et al., 1999**) has shown that *Rhodotorula* spp. was killed within 45 min, when exposed to the peroxide based sanitizer.

**Table 2** Determination of inhibitory activity of 1% and 3% aqueous solution of Bioxil against *S. cerevisiae* J-200 by suspension method

| Bioxil | Initial quantity of cells in suspension, cfu/ml (N) | Incubation time, min | Quantity of cells after testing ($N_a$), cfu/ml | Reduction in viability, cfu/ml (R) |
|---|---|---|---|---|
| 1% $H_2O_2$ | 3 x $10^6$ | 5 | 1.1 x $10^4$ | > $10^2$ |
| | | 15 | 4 x$10^3$ | < $10^3$ |
| | | 30 | 3 x $10^3$ | $10^3$ |
| | | 60 | 2 x $10^3$ | >$10^3$ |
| 3% $H_2O_2$ | 2 x $10^6$ | 5 | 2 x $10^3$ | $10^3$ |
| | | 15 | 4 x $10^2$ | < $10^4$ |
| | | 30 | 2 x $10^2$ | $10^4$ |
| | | 60 | 100 | > $10^4$ |

Direct relation between efficacy, concentration and contact times of Bioxil was established. 3log and 4log reduction of *S. cerevisiae* J-200 has been occurred after 60 min of contact time in case of 1% and 3% of $H_20_2$ respectively. **Aarnisalo et al., (2000**) also confirmed that reductions of the studied bacteria and yeasts increased with time of exposure and the disinfectant concentration.

Antifungal activity of the 3% solution of Bioxil against mix culture of *R. rubra, S. cerevisiae* and *Kloeskera* spp. has been studied in laboratory conditions by suspension method, (Tab 3). Mentioned yeast species are constantly form biofilms on conveyors /belts/ and on processing equipment.

**Table 3** Antifungal activity of the aqueous solution of Bioxil (concentration of $H_2O_2$ is 3%) against mix culture of *R. rubra, S. cerevisiae* and *K. apiculata* , by suspension test

| Bioxil | Initial quantity of cells in suspension, cfu/ml (N) | Incubation time, min | Quantity of cells after testing ($N_a$), cfu/ml | Reduction in viability, cfu/ml (R) |
|---|---|---|---|---|
| 3% $H_2O_2$ | 2 x $10^8$ | 5 | 6 x $10^5$ | < $10^3$ |
| | | 15 | 5 x $10^5$ | $10^3$ |
| | | 30 | 2 x $10^6$ | < $10^3$ |
| | | 60 | 7 x $10^6$ | < $10^2$ |

Two log reduction of viable yeast cells has been noticed at the end of 60 minutes of contact time with the tested biocide. Therefore, the mix culture of yeasts showed significantly higher resistance to 3% aqueous solution of Bioxil. High level of contamination of the environment as well as simultaneous presence of the different yeast species increases the resistance of yeasts to biocides (**Korukluoglu et all., 2006**). The resistance of the yeast species is attributed to their thicker cells walls (**Fleet, 1990).**

The hydrogen peroxide based sanitizer proved to be the most effective inhibitor against all the yeasts, resulting in final counts ranging from zero to $3x10^3$ cfu/ml after 60 min of contact. Poor killing effect of hydrogen peroxide were attributed to low concentration or too short contact time, about 10 min (**Bundgaard-Neilsen & Neilsen, 1995).**

The presence of organic matter or hard water reduces the effectiveness of disinfectants (**Holah, 1995**), and this is considered in the disinfectant efficacy tests proposed by the European regulation UNE-EN-1650. In our work, 1.0% (v/v) of strawberry juice has been used to simulate the practical conditions of the juice processing. Treatment of artificially contaminated stainless steel coupons with the Bioxil without preliminary washing by caustic soda has shown significantly lower DR /<2log/. 4log reduction of viable *S. cerevisiae* has been occurred at the end of 30 minutes of treatment of stainless steel coupons by 3% aqueous solution of Bioxil with preliminary washing by caustic soda. Results are shown in the figure 1. In consequence, to assure effectiveness of the applied disinfectant, it is still extremely important to clean in advance, thoroughly and regularly the equipment used for food processing (**Luppens et al., 2002; Taormina & Beuchat, 2002**).

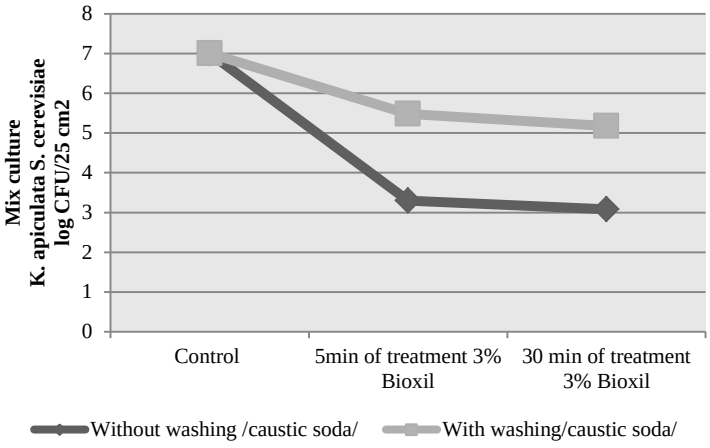


**Figure 1** Efficacy of 3% solution of Bioxil on stainless steel coupons by surface test

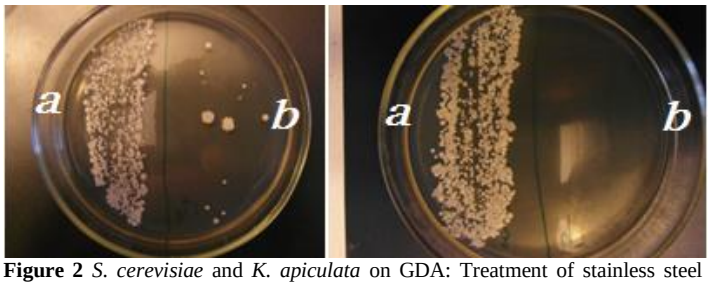


**Figure 2** *S. cerevisiae* and *K. apiculata* on GDA: Treatment of stainless steel coupons by 3% solution of t*he Bioxil (a-* before, *b-* after *treatment) : with (left) and without(right) prewashing by caustic soda*

Testing of the Bioxil has been performed also in processing conditions after washing with caustic soda and without preliminary washing. The results are shown in figure 3.

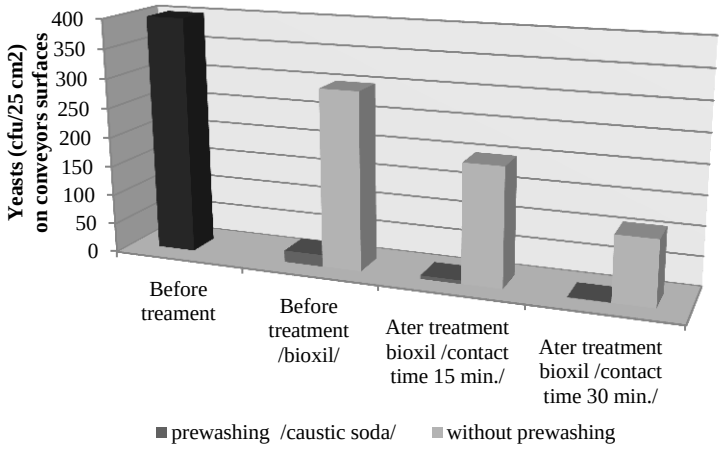


**Figure 3** Influence of the Bioxil /with and without prewashing by caustic soda/ on yeasts contaminating surfaces of conveyors in processing conditions

*S. cerevisiae*, *R. rubra* and *K. apiculata* were isolated from the surface of conveyor in predominant quantities. Treatment of the conveyor surfaces with the Bioxil directly after production process has shown 1 log reduction of yeasts at 15 and 30 minutes of treatment time. Preliminary washing of conveyors surfaces by 5% solution of caustic soda increases the efficacy of the Bioxil and leads to 4 log reduction of yeasts. Results obtained in processing have been confirmed by the results in laboratory conditions.

## CONCLUSION

Results of the suspension test have demonstrated good antifungal activity of the Bioxil. However, its efficiency depends on the certain yeast species. Not stable activity has been revealed against *R. rubra.* The mix culture of *R. rubra, S. cerevisiae* and *Kloeskera apicualata* has shown more resistance to mentioned disinfecting agent. Preliminary washing of processing equipment and working surfaces increased the efficiency and stability of the Bioxil.

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